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1. Your reference — P34693-/JDU/BOU

2. Patent application number *(The Patent Office will fill in this part)* — 24 SEP 2003 — 0323043.0

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Lux Biotechnology Limited
ETTC, Altrick Building, King's Buildings
Edinburgh
EH9 3JL
UK

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation — 8576845001

4. Title of the invention — "Biochip"

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

~~Murgitroyd & Company~~ RGC Jenkins & Co
~~165-169 Scotland Street~~ 26 Caxton Street
~~GLASGOW~~ LONDON
~~G5 8PL~~ SW1H 0RJ

Patents ADP number *(if you know it)* — ~~1198013~~ 11980015 / 03966736001

FS1/77 9/14

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
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See note (d))

Yes

Patents Form 1/77

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Description	15
Claim *(s)*	-
Abstract	- DL
Drawing *(s)*	5 + 5

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Priority documents	-
Translations of priority documents	-
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	-
Request for preliminary examination and search *(Patents Form 9/77)*	-
Request for substantive examination *(Patents Form 10/77)*	-
Any other documents *(please specify)*	-

11. I/We request the grant of a patent on the basis of this application.

Signature: Murgitroyd & Co

Date: 23 September 2003

Murgitroyd & Company

12. Name and daytime telephone number of person to contact in the United Kingdom: BEVERLEY OUZMAN 0141 307 8400

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Biochip

The present invention concerns a novel microdevice (biochip) for screening a plurality of biomolecule-analyte interactions.

A biochip may be defined as a collection of miniature test sites onto which a number of biomolecules are attached with high density and in a defined microarray on a solid surface such as a silicon wafer. With a typical size of $1cm^2$, the biochip enables simultaneous tests to be conducted, facilitating high throughput of testing.

Many biomolecules are active only in solution or in the presence of a second molecule. However often the activated form of the biomolecule has a finite useful lifespan, thereby curtailing the shelf-life of any biochip containing it. In particular the need for water and nutrients to maintain viability has limited the use of micro-organisms (such as bacteria or fungi) in biochips.

The present invention concerns a biochip able to store a first biomolecule separately to a second molecule able to activate it, but wherein the first biomolecule and second molecule can be selectively mixed together to cause the first biomolecule to be activated when the biochip is required. This design of biochip has the advantage that the first biomolecule may be stored in an inactive form providing a longer shelf-life for the biochip.

The present invention provides a microdevice comprising a plurality of individual chambers, wherein at least one of said chambers contains a first reactant in non-active form, a second reactant able to activate said first reactant when mixed therewith, wherein said first and second reactants are separated from each other by a separating means, wherein said separating means is displaced or perforated by laser activation thereby allowing said first and second reactants to be mixed together within said chamber and causing said first reactant to be activated.

The first reactant may be a micro-organism present in inactive form, for example as a spore. Mention may be made of fungal spores in this regard, but bacterial spores or other inactive forms of bacteria may also be used in the biochip. In this embodiment, the second reactant may be water, or may be a mixture of water and nutrients (e.g. sugars, amino acids, and/or metal ions) required to stimulate activation of the micro-organism.

Alternatively, the first reactant may be a protein or nucleic acid which requires the second reactant for activation. For example certain enzymes require the presence of a co-factor (e.g. metal ions, ATP, ADP or the like) for activity and these combinations would be suitable for use in the present invention.

To specifically inject a single chamber, a mechanism has been devised which allows accurate dispensing of liquid so that luminescence may be measured simultaneously. This site-specific injection is achieved using a novel method of laser stimulated injection. A laser beam is directed to a site on the chip composed of a light absorbing material which expands rapidly. Adjacent to this site is a chamber containing the fluid to be injected. Expansion of the laser-irradiated spot results in expansion of the material pushing the liquid into the chamber. The pressure of fluid breaks the temporary seal of the separating means which prevents the liquid flowing into chambers prematurely. The advantage of this method is that the biochip does not require electronic wiring and the use of a laser to activate individual chambers means that highly accurate results can be obtained without perturbing the samples. Other mechanisms for specific injection of fluid are described in Figure 3. Figure 3 shows different possible designs for laser activation.

In Figure 3a) the substrate is held within a membrane "bubble" under tension. The membrane acts

as the separating means. The laser ruptures the membrane and its contents are released.

In Figure 3b) the substrate is held within a separate chamber with a pressure sensitive seal. The pressure sensitive seal acts as the separating means. The laser causes heat sensitive material in the separate chamber to expand, rupturing the seal pushing reactant into the biosensor chamber containing the other reactant.

In Figure 3c) the first or second reactant is held within a cylindrical chamber, and a seal prevents contents mixing. The laser causes heat-sensitive material to expand, breaking the seal, and pushes the first or second reactants into the biosensor chamber containing the other reactant in similar way to a hypodermic syringe.

In Figure 3d) the first and second reactants are each held within a separate chamber and the connection between the chambers is sealed by a piston which acts as the separating means. The piston is anchored in a heat-sensitive material. The laser causes the heat-sensitive material to expand, pushing the piston, opening the seal, and mixing the contents of two chambers.

The use of the biochip with an imaging system is shown in Figure 4. It is envisaged that the biochip may be mounted onto plastic cassettes that fit into

the test chambers of commercially available luminometer or fluorometer equipment.

As mentioned above, the mixing of the first and second reactants is achieved by displacement or perforation of a separating means through use of a laser. The accuracy of focus achievable with a laser beam enables predetermined chambers within the biochip to be selectively activated and this ability to select just specific chambers for activation represents a significant advance in the art.

The separating means may be a membrane which is directly perforated by the laser. Alternatively, the laser may be focused onto a light absorbing material which expands to either cause rupture of the separating means or its displacement, sufficient to allow mixing of the first and second reactants.

In one embodiment the first reactant is a fungal spore immobilised onto the chamber. The spores may be held in a matrix which is easily wettable to achieve fast activation. Test substances may be added onto the chip using array spotter or inkjet technology. The chip is then sealed to retain moisture within the chambers.

The biochip may be formed from any suitable base material, typically a silicon wafer. Other base materials which may be contemplated include silicon dioxide, indium fin oxide, alumnia, glass and

titania. Moulded plastics or ceramics may also be suitable.

Generally the base material is micro-machined to have the desired configuration of chambers and channels. Micro-machining may be carried out using techniques known in the art or in the related art of semi-conductor and electronics manufacture, for example, laser ablation, electrodeposition, vapor deposition, chemical etching, dry etching, photolithography and the like. In its simplest form the biochip may comprise a grid pattern of separate chambers etched onto a silicon wafer.

The first and second reactants, and any other ingredients to be contained within the chamber, may be located onto all or any of the chambers on the pre-micro-machined base material. Known techniques such as ink-jet technology may be used for accurate placement of pre-determined aliquots of each ingredient/reactant. Optionally an adhesive substance may be used to ensure retention of the first and/or second reactant, and such adhesive substance may be applied prior to the introduction of the first and/or second reactant, simultaneously with the first and/or second reactant or subsequently as a covering layer.

In one embodiment, the separating means is located in each chamber dividing the chamber into two parts (which may be the same or of different

areas/volumes) prior to introduction of the first and second reactants.

Alternatively, the separating means may be applied as a layer on top of either one of the first or second reactants, which reactant has already been located in the chamber. The other of the first or second reactants may then be located on top of the separating means prior to the biochip being sealed.

Once the first reactant, the separating means and the second reactant have been located in the biochip, the biochip is sealed with a suitable outer layer. The outer layer should be strong enough to withstand damage and should also prevent leakage and evaporation. Mention may be made of nitro-cellulose or polypropylene as being suitable materials.

A preferred first reactant are fungal spores, in particular spores of filamentous fungi. Suitable fungi include *Aspergillus sp*. and *Neurospora sp*. A yeast such as *Saccharomyces cerevisiae* may also be used.

Optionally the fungi will have been bio-engineered to luminesce or fluoresce in the presence of a pre-selected analyte.

Optionally, the luminescence output varies in response to the presence or absence of the pre-selected analyte.

Optionally the luminescent protein is a foreign protein and the filamentous fungi is genetically engineered to express that protein and to be luminescent, by introduction of the relevant gene.

The gene for a luminescent protein may be obtained from firefly (*Photinus pyralyis*), crustaceans (*Cyridina hilgendorfi*), dinoflagellates (*Nortilucus militaris, Gonyaulax polyhedra*) or naturally luminescent fungi (*Panellus stipticus*). Use of luminescent proteins of bacterial origin are also possible.

Preferred luminescent proteins include luciferase proteins, for example from *Gaussia*. Suitable genes expressing luminescent proteins are described in WO-A-99/49019.

Suitably the *Gaussia* luciferase is genetically engineered into *Neurospora crassa*, and optimised for mammalian codon usage. This mammalian gene can be successfully expressed in filamentous fungi.

Gaussia luciferase may be expressed in other species of filamentous fungi including *Aspergillus nidulans* and *Sclerotinia sclerotiorum* (a plant pathogen). *Gaussua* luciferase gene may be codon-optimised for codons preferred by filamentous fungi in order to increase light output. Other novel luminescent and fluorescent proteins (e.g. the calcium-sensitive *Obelin* photoprotein, and the *Ptilosarcus* green

fluorescent protein) may also be expressed in filamentous fungi.

In the biochip, the luciferase may be expressed in response to specific stimuli (particularly the presence of sodium ions) by driving the luciferase expression with inducible promoters. The alcA promoter is induced in response to ethanol utilisation (see Felenbok B (1991) "The ethanol utilisation regulation of *Aspergillus nidulans* the alcA-alcR system as a tool for expression of recombinant proteins". *Journal of Biotechnology* 17: 11-18; Flipphi M, Kocialkowska J, Felenbok B (2002) "Characteristics of physiological inducers of ethanol utilisation (*alc*) pathway in *Aspergillus nidulans*". *Biochemical Journal* 364: 25-51).

The alcA promoter has been used to drive expression of Green Fluorescent Protein (GFP) in *Aspergillus nidulans* (see Fernández-Ábalos JM, Fox H, Pitt C, Wells B and Doonan JH (1998) "Plant adapted green fluorescent protein is a versatile reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*". *Molecular Microbiology* 27: 121-130). Transformation of *Aspergillus nidulans* with luciferase genes may be fused to the alcA promoter. The copper metallothionein is expressed in response to copper ions and thus could form the basis of a copper biosensor through the expression of luciferase fused to the copper metallothionein promoter from *Neurospora crassa* (see Munger K, Germann UA, Lerch K

(1985) "Isolation and structural organisation of the *Neurospora crassa* copper metallothionein gene". *EMBO Journal* 4: 2665-2668; and Schilling B, Linden RM, Kupper U and Lerch K (1992) "Expression of *Neurospora crassa* Laccase under control of the copper inducible metallothionein promoter", *Current Genetics* 22: 197-203).

The expression of the luminescent protein is desirably under the control of a gene promoter or enhancer sensitive to the presence of the pre-selected analyte to be assayed in the biochip.

The pre-selected analyte is suitably sodium ions, organophosphate, alcohol or copper ions.

The present invention also provides a method of detecting an analyte in a sample, said method comprising providing a biochip as described above wherein said first reactant in activated form is able to luminesce in the presence of said analyte; focussing a laser beam onto an expandable material located adjacent the separating means, thereby causing expansion of said expandable material and displacement or rupture of said separating means; retaining said biochip at a suitable temperature to facilitate activation for at least one hour; introducing said sample to said biochip; and measuring the luminescent output.

The present invention also provides a microdevice comprising a plurality of individual chambers

wherein at least one chamber contains a first reactant in non-active form, and wherein prior to use of the microdevice a second reagent able to activate said first reagent when mixed therewith is introduced into each chamber containing said first reactant, thereby causing said first reactant to be activated.

In one embodiment, the chambers containing said first reactant are connected by a series of channels, and said second reactant is caused to flow along said channels and into the chambers containing said first reactant. Optionally said second reactant may be introduced into the chambers under pressure.

Injection of the liquid into the biosensor chambers can be accomplished in different ways. To activate all chambers, the liquid is injected through channels which connect with all or a selected group of chambers on the array. The flow of liquid may be regulated by allowing it to flow through an absorbent material ensuring uniform distribution. Following addition of the growth medium, the chip is sealed and incubated for between 4 and 24 hours.

The present invention will now be further described with reference to the following non-limiting examples and figures in which:

Figure 1 is a schematic diagram showing the arrangement of a prototype capillary tube laser

activated pump. During laser irradiation liquid is pushed along the tube.

Figure 2 shows photographic images of a capillary tube laser activated pump at 1 second (1s), 30 seconds (30s) and 60 seconds (60s) of irradiation with a 870 nm laser beam.

Figure 3 shows alternative designs for a laser activated chamber within the biochip of the invention.

Figure 4 is a schematic diagram showing the use of the biochip of the invention within an imaging system.

Figure 5 (a) Cellulose membrane coated with spores of *Neurospora crassa* (Bar = 1 mm). (b) Cellulose membrane after placing on agar for 24 hours results in germination of spores and formation of mycelial colonies (Bar = 1 mm).

Figure 6 Biochip populated with germinating spores of *Neurospora crassa*. Spores were hydrated for 2 hours and show growth. (Bar = 100 μm).

Example 1

Laser irradiation of distilled water containing activated charcoal particles.

A liquid consisting of 10mg activated charcoal per ml distilled water was drawn into a glass capillary tube of 1 mm outer diameter, 0.58 mm inner diameter. The activated charcoal was used since it possesses a dark colour which absorbs the maximum amount of light. One end of the capillary was sealed. The loaded capillary was placed in the stage of an inverted microscope and imaged using a X10 Plan Apo objective (NA = 0.45). The multi-photon system consisted of a Bio-Rad Radiance 2100 with a coherent Mira Ti-Sapphire laser tuned to 870 nm. The laser was used a full power and scanned for 50 x 2-second pulses. Upon irradiation, the laser energy caused the water to heat up, and boil. The boiling created water vapour, which pushed the liquid along the capillary tube. A schematic illustration of the experiments is illustrated in Figure 1.

Figure 2 shows images of the capillary tube at 1s, 30s and 60s of laser irradiation. At 30s, 0.195µl of water has been pushed along the tube. After 60s, 0.298µl of water has been pushed along the tube. The irregular black lines with the water are moving particles of activated charcoal. The movement of the water clearly demonstrates that a laser can be used to cause a flow of liquid sufficient to facilitate mixing of the first and second reactants in a chamber of the biochip.

Example 2

Manufacture of a biochip containing fungal spores as a first reactant.

Spore immobilisation

Cellulose membrane (cellophane) was cut into squares of 1.5mm x 1.5mm. The membranes were then moistened with distilled water and sterilised in an autoclave. Spores of *Neurospora crassa* were harvested and suspended in a solution of 5% milk and 2% glutamic acid. The spore solution was then added to the cellulose squares, coating them with spores (Figure 5a). The cellulose squares were then placed in a Petri dish and dried in a laminar flow hood for 2 hours. After 2 weeks storage at 20°C the spore-coated squares were then placed on malt extract agar and incubated for 24 hours. After microscopic examination, it was noted that germination had occurred and mycelial colonies were developed (Figure 5b).

Prototype biochip

Nitrocellulose (pyroxylin) was dissolved in absolute ethanol and painted onto a silicon (approx 1.5 cm^2) wafer with electron-beam etched squares of 100 µm x 100 µm and 0.5 µm height. The nitrocellulose was allowed to dry for 20 minutes and then peeled off the silicon wafer. This process resulted in a "negative" imprint of the silicon wafer consisting of 100 µm square wells of 0.5 µm deep. Spores were then deposited on the surface of the chip.

Polylycine may be sprayed onto the chambers prior to introduction of spores. The polylycine acts as an adhesive to retain the fungal spores which may be accurately placed into each chamber using ink-jet technology. Between 1 and 100 spores may be located per chamber. The biochip was dried in a laminar flow hood at 25°C. The drying process was complete within 1-5 minutes thus ensuring that the spores remained dormant. For activation, the entire chip was then hydrated with 20 µl of distilled water. The chip was inverted and placed onto a coverslip (sandwiching the spores between the cellulose and glass). After 2 hours the sample was examined on a microscope and germination had occurred (Figure 6). Spores were subsequently observed over a period of 4 hours, and exhibited normal growth.

Several biochip layers may be combined, each may contain growth media and substrates (e.g. coelenterazine) or fluorescent probes (e.g. propidium iodide, FM4-64). When use of the biochip is required, separating layers may be perforated by focusing a laser beam onto them. The rupture of the separating layer enables the growth medium (or other solution containing substrates e.g. coelenterazine) to flow into the lower compartment containing the dormant spores. The spores will be activated following between 1 to 24 hours incubation at ambient temperature and the biochip will be ready for use. The biochip can be stored for several months without deterioration.

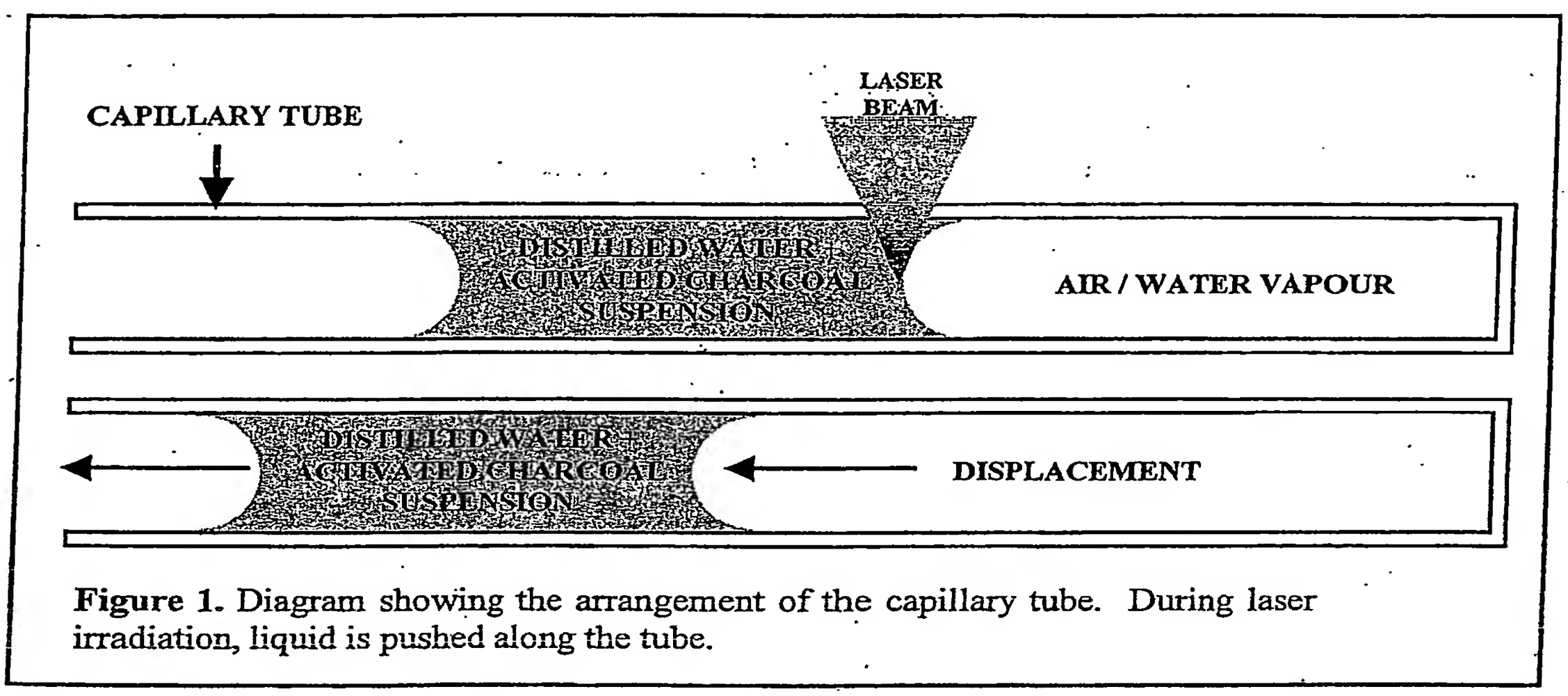


Figure 1. Diagram showing the arrangement of the capillary tube. During laser irradiation, liquid is pushed along the tube.

Figure 2. Images of the capillary described above. The sample was irradiated with 870 nm laser beam and imaged using a confocal microscope. After 30 seconds, 0.195 µl of water has been pushed along the tube. After 60 seconds, 0.298 µl of water has been pushed along the tube. The irregular black lines within the water represent moving particles of activated charcoal.

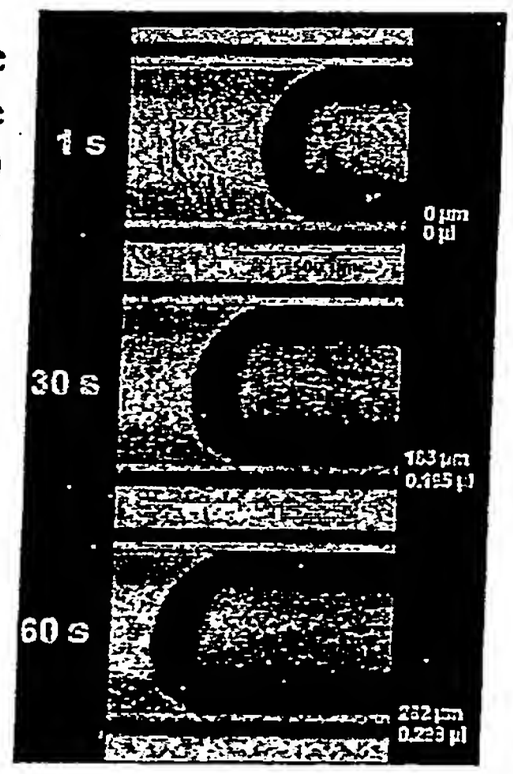
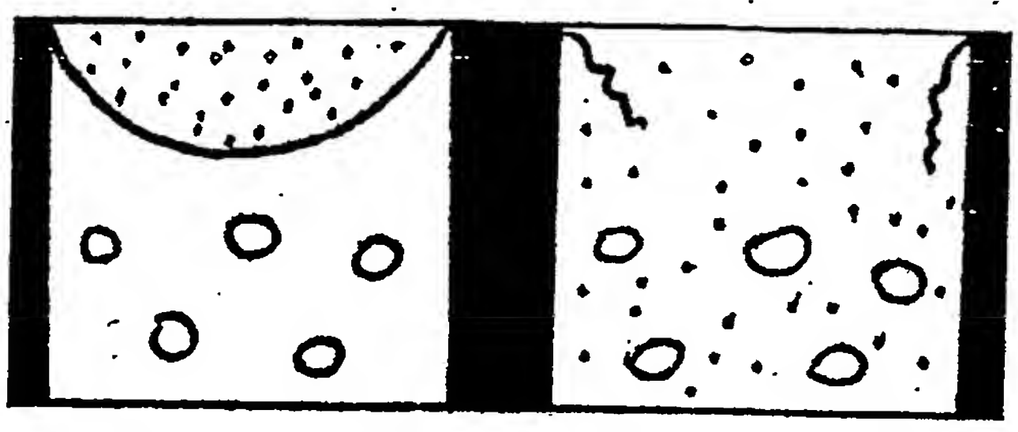
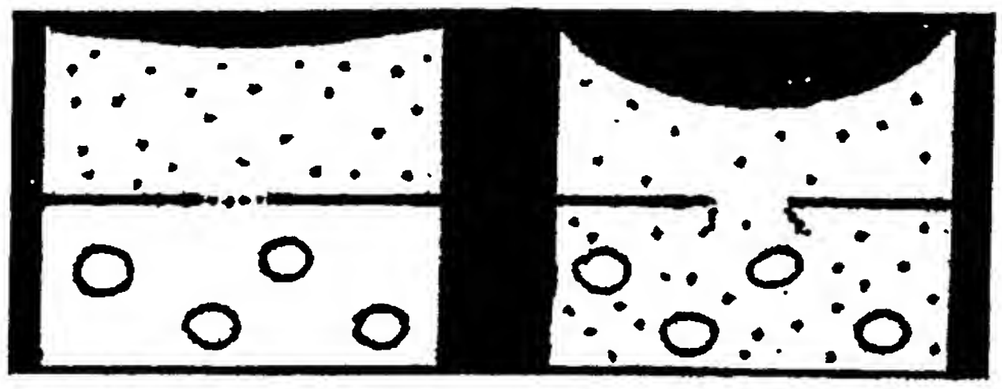


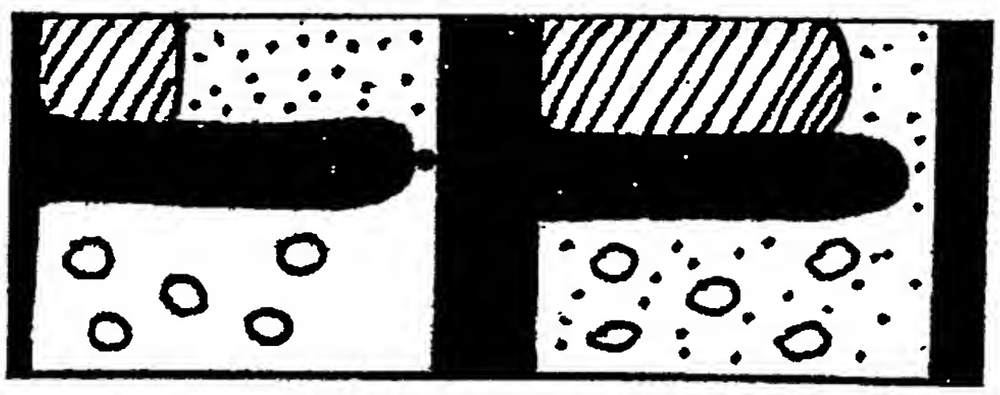
Figure 3. Design for laser-activated chambers.



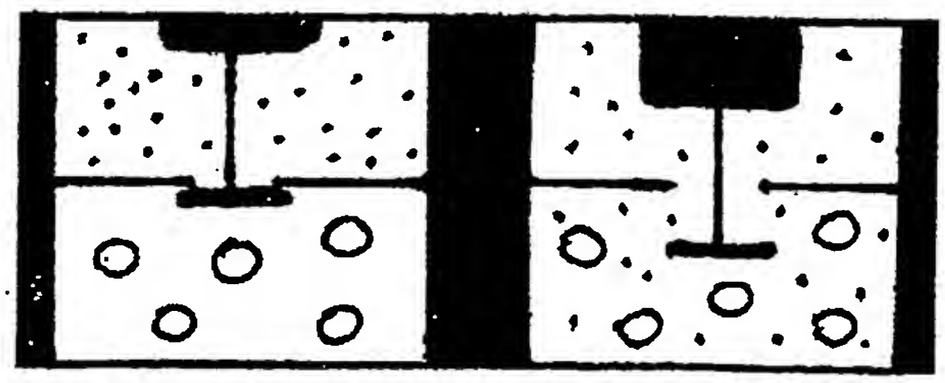
(a) The substrate is held within a membrane "bubble" under tension. The laser ruptures the membrane and contents are released.



(b) The substrate is held within a separate chamber with a pressure sensitive seal. The laser causes heat sensitive material in substrate chamber to expand, rupturing the seal pushing substrate into biosensor chamber.



(c) The substrate is held within a cylindrical chamber, and a seal prevents contents mixing. The laser causes heat-sensitive material to expand, breaks seal, and pushes substrate into biosensor chamber in similar way to a hypodermic syringe.



(d) The substrate is held within a separate chamber and sealed by a piston. The piston is anchored in a heat-sensitive material. The laser causes the heat-sensitive material to expand, pushing the piston, opening the seal, and mixing the contents of two chambers.

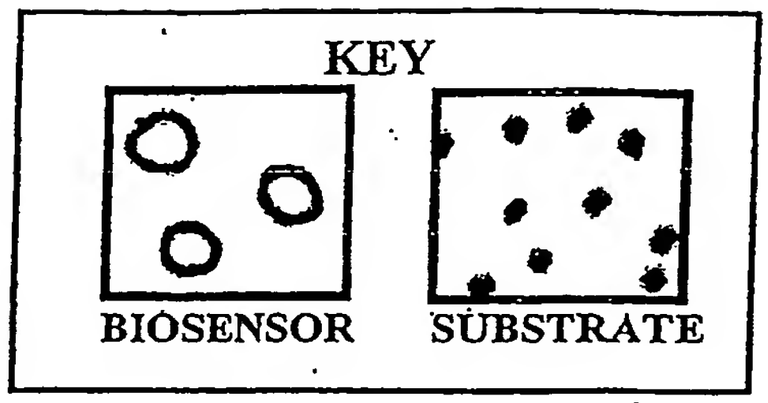
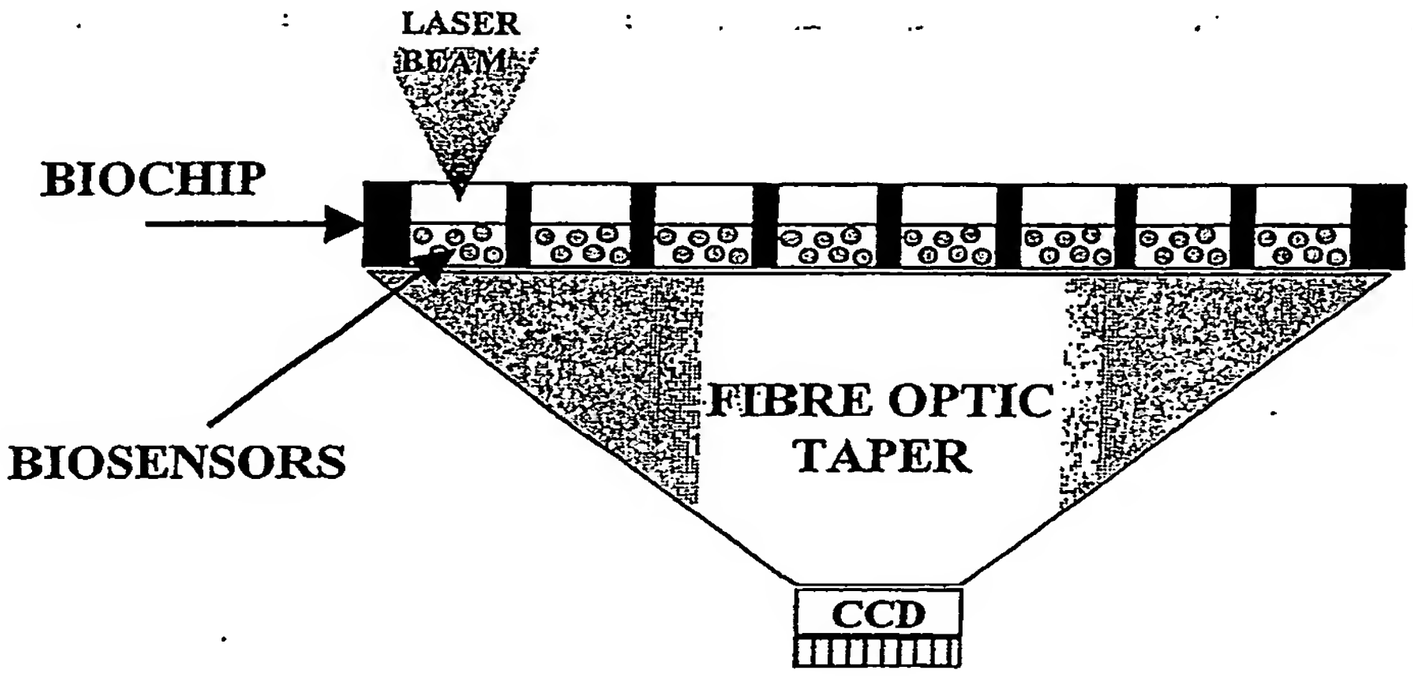
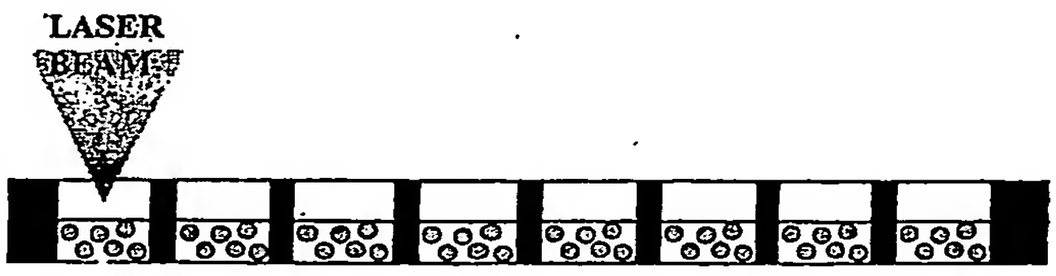
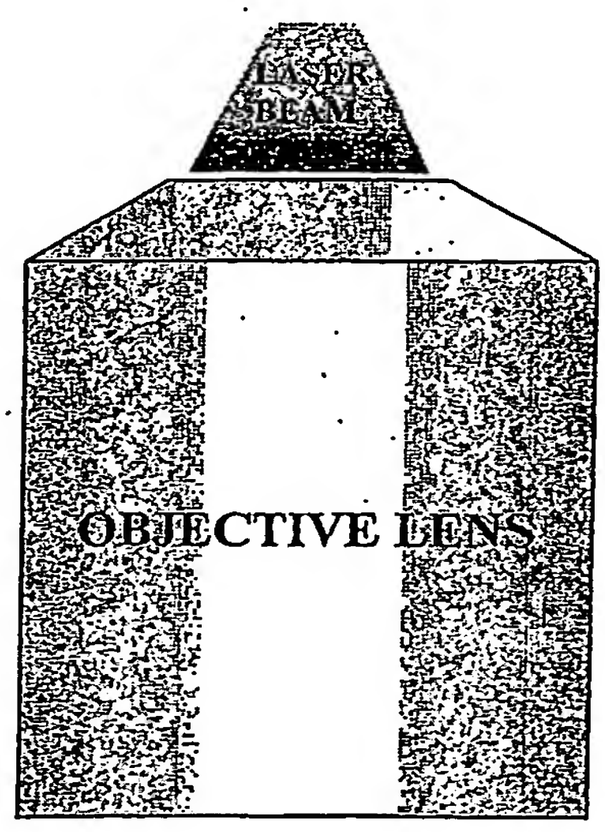


Figure 4. Use of the biochip within an imaging system. The biochip is designed to be imaged, either using a contact-imaging device such as a CCD chip coupled to an optical taper, or an inverted microscope. The laser beam can be directed using the same lens of an inverted microscope, or it can be applied from the opposite side.







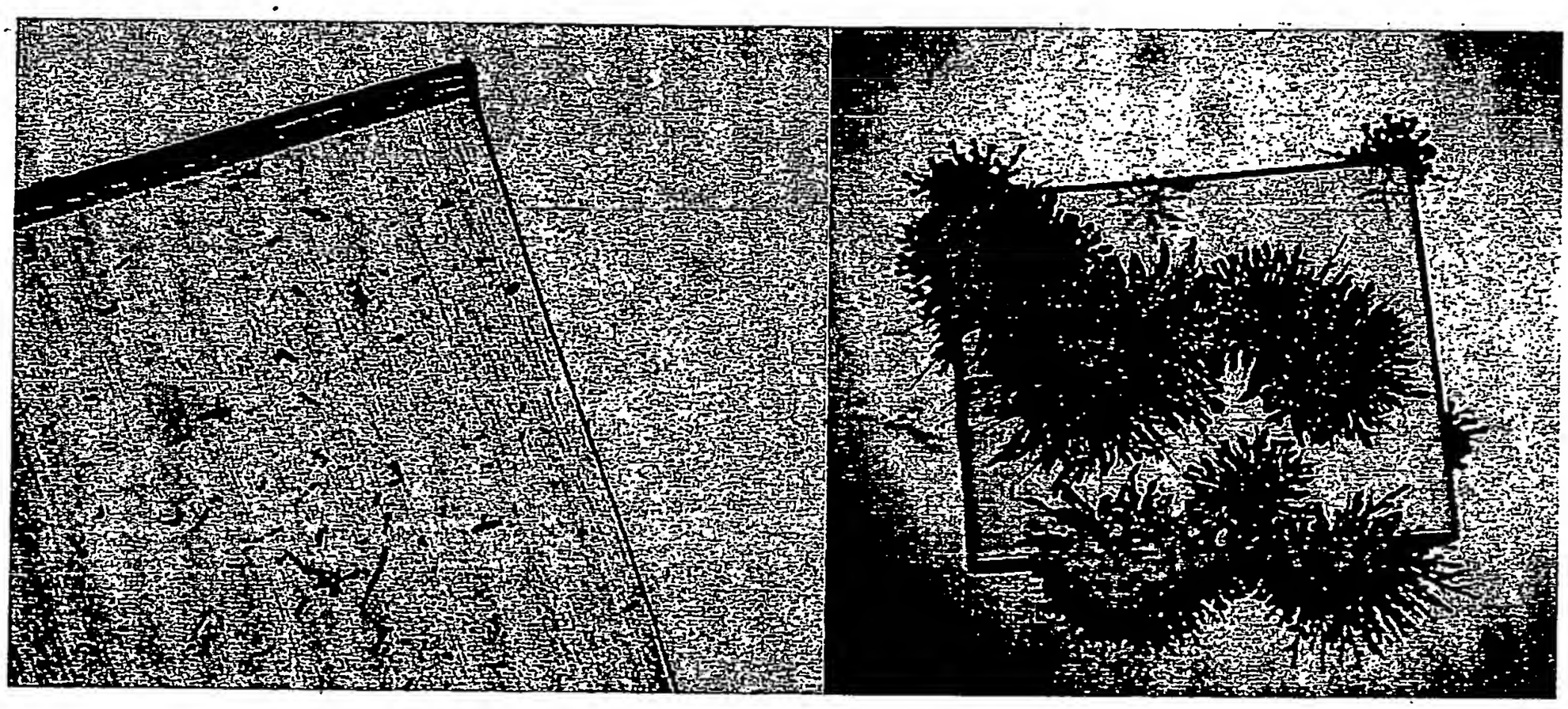


Fig. 5

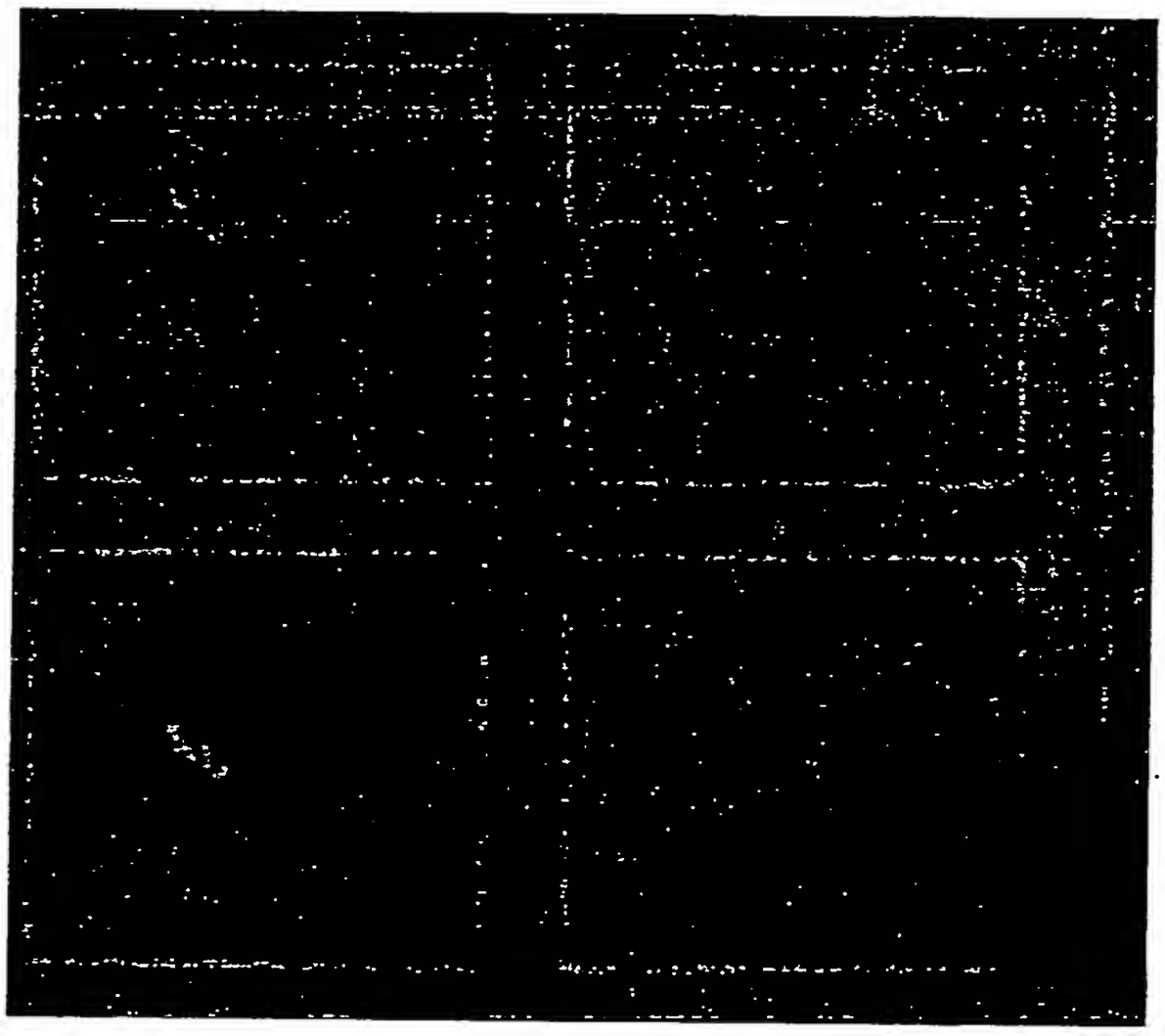


Fig. 6